Effects of Castration on *Androgen Receptor, IGF-I Ea, MGF* and *Myostatin* Gene Expression in Skeletal Muscles of Male Pigs

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ABSTRACT : Castration of male pig produces significant negative effects on skeletal muscle development. The *androgen receptor* (*AR*), two splice variants of *insulin-like growth factor-I* (*IGF-I Ea* and *MGF*) and the *myostatin* gene may play important roles in this process. In the present study, the expression of *AR, IGF-I Ea, MGF* and *myostatin* genes in three skeletal muscles, the brachialis, longissimus and semitendinosus, were studied using real-time quantitative RT-PCR. Our experimental design used 14 pairs of male Landrace sire×Yorkshire dam piglets. The two piglets in each pair were full sibs, one of which was castrated at 21 d of age; the other remained intact. The study group was divided into subgroups of equal size. Animals in the first subgroup were slaughtered at 147 d and those of the second at 210 d of age. Carcass weight and lean meat yield were similar between boars and barrows at 147 d of age (p>0.05), whereas barrows had lower carcass weight and less lean meat yield at 210 d of age (p<0.05). Castration caused down-regulation of *AR* gene expression at both 147 and 210 d of age (p<0.05). The two splice variants of the *IGF-I* gene from porcine skeletal muscle were cloned using RT-PCR, and it was found that *MGF* differs from *IGF-I Ea* in having a 52-base insert in the last coding exon of the mRNA. Both splice variants were down-regulated by castration only at 210 d of age (p<0.05). No differences in expression of the *myostatin* gene were observed between boars and barrows at either 147 or 210 d of age (p>0.05). These results suggest that the down-regulation of *AR, IGF-I Ea* and *MGF* gene expression following castration helps to explain the negative effect of castration on skeletal muscle development. (**Key Words** : Pig, Castration, Skeletal Muscle, Molecular Cloning, Gene Expression)

INTRODUCTION

It is well known that physiological differences exist between intact male pigs and castrates. Compared to barrows, boars have better growth performance, more lean meat and less fat accumulation in the carcass. However, the molecular mechanisms underlying the effects of castration on porcine skeletal muscle development remain unclear and need to be studied more closely at the molecular level.

Investigation of genes expressed during skeletal muscle development is elementary in understanding molecular mechanism of muscle growth and can contribute to the discovery of candidate genes associated with meat production and quality traits (Chen et al., 2008).

Androgen exerts its function through the androgen receptor (AR). Androgen-bound AR acts as a transcription factor, which regulates genes involved in many physiological processes. It has been proven that the AR and its co-regulatory proteins are pivotal factors in skeletal muscle differentiation (Wannenes et al., 2008).

Insulin-like growth factor-I (IGF-I) is a ubiquitous peptide that plays important roles in both embryonic and postnatal development of skeletal muscle. It has been shown that androgens may exert an effect on skeletal muscle growth through local IGF-I. There are two splice variants of the *IGF-I* gene expressed in skeletal muscle, *IGF-I Ea* and *mechano growth factor* (*MGF*), and these different *IGF-I* splice variants have been shown to have different functions. IGF-I Ea promotes an increase in cellular mass and induces myoblasts to fuse and form myotubes, while MGF increases cellular proliferation and inhibits terminal differentiation to produce more myoblasts

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for secondary myotube formation and to establish a satellite (stem) cell pool (Cheema et al., 2005). It is not known how splice variants of the *IGF-1* gene interact with androgens in skeletal muscle development.

Myostatin, a member of the transforming growth factor beta (TGF-β) superfamily, is a specific negative regulator of skeletal muscle development and growth. Putative androgen response elements are present in the 5'-regulatory region of the human *myostatin* gene promoter (Ma et al., 2001). Thus, castration could also affect *myostatin* gene expression in skeletal muscle.

The objective of this study was to investigate variations in the expression of *AR, IGF-1 Ea, MGF* and *myostatin* genes in the skeletal muscles of male pigs after castration. We cloned *IGF-1 Ea* and *MGF* cDNA from porcine skeletal muscle using RT-PCR. Expression of these genes was quantified using real-time quantitative RT-PCR using samples taken from three skeletal muscles, brachialis (BR), longissimus (L) and semitendinosus (ST) from boars and barrows. This study aimed to elucidate the molecular mechanisms in which castration produces negative effects on skeletal muscle development, and to provide insights into the mechanism of actions of anabolic steroids in skeletal muscle growth and development.

MATERIALS AND METHODS

Animals

Animals were managed under normal husbandry conditions and all procedures were approved by the local Animal Care Committee. Fourteen pairs of full sibs, a total of 28 male Landrace sire×Yorkshire dam piglets, were selected at 20 d of age from animals kept at the Zhejiang University breeding farm. All pigs were weaned at 30 d of age. At 21 d of age, both testicles of one piglet in each pair were removed by surgical castration under anesthesia; the second piglet in each pair remained intact. Three diets according to live weight were used during the growth phase. The daily feed was as follows: 63.0, 64.0 and 65.0% maize; 7.0, 10.0 and 14.0% wheat bran; 22.0, 15.0 and 10.0% soybean meal; 2.0, 3.0 and 5.0% rapeseed meal; 5.0, 4.0 and 2.0% fish meal during three phases of live weight: <50 kg, between 50 and 80 kg and >80 kg, respectively. All pigs were allowed *ad libitum* access to feed and water until slaughter.

Carcass evaluation

Seven pairs of full sibs were slaughtered at 147 d of age while the remaining full sibs were slaughtered at 210 d of age. Before slaughter, all animals were fasted for 24 h. Immediately after stunning and exsanguination, pigs were dehaired and eviscerated. The head was removed and the carcass was split longitudinally. Weights of left and right sides were recorded and the left side of each carcass was physically dissected into bone, muscle, fat and skin. The weight of each component was recorded. Longissimus muscle area was determined by tracing its surface area at the 10th rib.

Tissue collection

The BR, L and ST muscles were removed immediately after slaughter, frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

Total RNA isolation and cDNA synthesis

Total RNA from BR, L and ST muscles was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was quantified at 260 nm using the NanoDrop spectrophotometer (NanoDrop ND-100, NanoDrop Technologies, Wilmington, DE, USA). The quality of total RNA was assessed by 1.2% (w/v) agarose gel electrophoresis, and samples showing a good RNA quality were selected for further reverse transcription. One microgram of total RNA was used for cDNA synthesis with the Improm-II First-Strand cDNA Synthesis Kit (Promega, Madison, USA) according to the manufacturer's protocol. The product was stored at -20°C until use.

Cloning and sequencing of porcine IGF-I splice variants

Based on GenBank sequences of the *IGF-1* gene (accession numbers: M31175, NM_214256), a pair of primers (Table 1) was designed to amplify the two splicing forms in porcine skeletal muscle. The PCR reaction was performed in a 25 μl total volume containing 1 μl of the reverse transcription product, 300 nM of forward and reverse primers, 0.4 mM dNTP, 10×reaction buffer, and 2 units Taq DNA polymerase (Tiangen Biotech, Beijing, China). The following PCR conditions were used: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were sequenced after cloning into the pMD18-T Vector (TaKaRa, Dalian, China).

Real-time quantitative RT-PCR

The primer information for the *AR, IGF-I Ea, MGF* and *myostatin* genes as well as for the housekeeping internal control gene (*glyceraldehyde 3-phosphate dehydrogenase, GAPDH*) are listed in Table 1. All primers were designed based on the sequences published in GenBank and using the OLIGO Primer Analysis Software v6.41. To prevent amplification of residual genomic DNA, at least one primer of each pair was positioned across an exon-exon boundary.

Table 1. Oligonucleotide primers used for cloning the porcine splice variants of *insulin-like growth factor-I* (*IGF-I*) gene and real-time quantitative RT-PCR

	Primer name	Primer sequences (5′ to 3′)	Product size (bp)
Clone	Clone forward	ATCACATCCTCTTCGCATC	548/600
	Clone reverse	GGGAATGTTTACTTGTGTATT	
Real-time quantitative RT-PCR	AR forward	TTCACCAATGTCAACTCCAGG	126
	AR reverse	TCCAAATTCTTGAGAGAGATGC	
	IGF-I Ea forward	ATCGTGGATGAGTGCTGC	143
	IGF-I Ea reverse	AAATGTACTTCCTTCTGAGCCT	
	MGF forward	CACACGGACATGCCCAAG	90
	MGF reverse	AAATGTACTTCCTTTCCTTCGC	
	Myostatin forward	TCCAACTTAGGCATTGAAATC	105
	Myostatin reverse	GACTTCTAAAAAGGGATTCAGC	
	GAPDH forward	GAAACCTGCCAAATATGATG	112
	GAPDH reverse	GTTAAAGTCAGAGGACACAACC	

Gradient PCRs confirmed 60°C as the appropriate annealing temperature for all primers. All of the target sequences were initially sequenced to ensure the specificity of the primers.

A standard curve-based method was used for data processing of real-time quantitative PCR, as described by Larionov (2005). Serial dilutions of standard plasmid DNA were included in each individual plate from which a standard curve was created. To obtain ideal standard curves, we used 6 five-fold dilutions for genes with relatively stable expression and 6 ten-fold dilutions for genes with less stable expression.

Real-time quantitative PCR was performed using an ABI PRISM 7900 instrument (Applied Biosystems, Foster City, CA, USA). The final reaction volume was 20 µl. Each reaction well was loaded with 9 µl SYBR Green mix (RealMasterMix, Tiangen Biotech, Beijing, China), 1.2 pmol primers (forward and reverse primer), 1 µl cDNA (made from 1 µg RNA) and nuclease-free water to make up the reaction volume. The following PCR conditions for all genes were used: 2 min at 50°C, 3 min at 94°C and 40 cycles of 30 s at 94°C, 40 s at 60°C and 20 s at 68°C. At the end of each run, dissociation melt curves were obtained, which enabled the specificity of the reaction to be determined. All samples, including the non-template control, were run in triplicate and mean values were subsequently used for analysis.

Statistical analysis

Standard curve and mRNA expression were analyzed using SDS 2.2 software (Applied Biosystems, Foster City, CA, USA). The relative mRNA levels were determined using real-time quantitative PCR and normalized using housekeeping gene *GAPDH* as an internal control. Statistical analysis was performed with SPSS software, 10.01 (SPSS Inc. Chicago, IL, USA). Data of average relative mRNA levels between boars and barrows were analyzed using paired-samples T-test. Data are presented as mean±SEM.

RESULTS AND DISCUSSION

Live weight and carcass characteristics

A plot of live weight data against age for the two sexes is presented in Figure 1. Boars were heavier than barrows at 210 d of age and the difference was significant (p = 0.031), but no sex-related differences were found at 84 or 147 d of age (p = 0.438, p = 0.688, respectively). The effects of castration on carcass characteristics of male pigs are shown in Table 2. The differences in growth performance and carcass characteristics between boars and barrows were consistent with other published reports (Castell and Strain, 1985; Knudson et al., 1985; Kumar and Barsaul, 1991; Sookhareea et al., 2001).

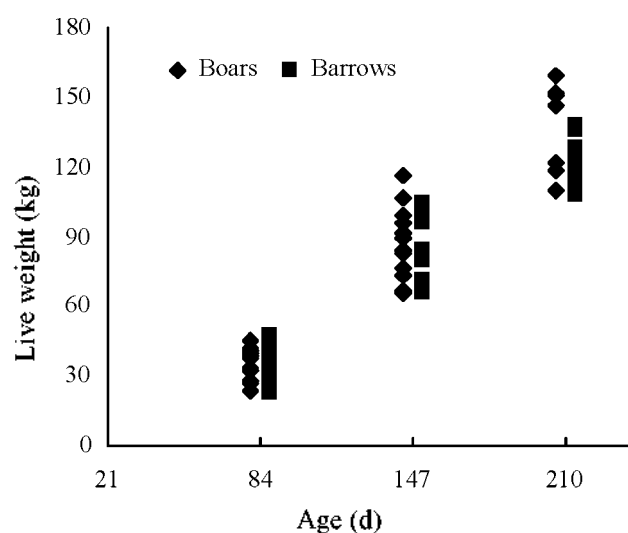These results indicated that castration of the male pig

Figure 1. Live weight of boars and barrows. The data showed that boars were heavier than barrows at 210 d of age (p = 0.031), but there were no differences between boars and barrows at younger ages.

Table 2. Effects of castration on carcass characteristics of male pigs

Items	147 d			210 d		
	Boars (n = 7)	Barrows (n = 7)	p-value	Boars (n = 7)	Barrows (n = 7)	p-value
Carcass weight (kg)	61.80±3.87	61.66±3.06	0.945	95.33±5.51	87.09±3.90	0.048
Carcass length (cm)	86.29±2.37	85.71±1.74	0.522	98.71±1.04	95.79±1.30	0.152
Lean meat yield[a] (kg)	19.06±1.57	18.11±1.49	0.142	27.81±1.39	23.54±0.92	0.006
Longissimus area (cm^2)	46.38±3.65	40.95±4.59	0.105	59.03±3.49	51.34±1.96	0.015
Lean meat percentage (%)	61.00±1.63	57.23±2.37	0.025	58.18±0.77	53.67±0.84	0.008

[a] The data were collected from left side carcass.

results in significant negative effects on skeletal muscle growth. This may partially reflect the earlier maturation of barrows compared with boars. It seems that superior muscle growth in intact males is manifested only when animals are approaching puberty. Castrated male pigs usually reach their lean growth potential and begin to deposit fat earlier compared with intact males.

The superior skeletal muscle growth of boars compared to barrows seems to be mainly due to the anabolic effect of gonadal steroids. Androgens have long been known to have positive effects on skeletal muscle growth. These effects are characterized by increased protein synthesis and accretion (Martinez et al., 1984; Mauras et al., 1994; Katznelson et al., 1996), increased satellite cell proliferation (Joubert et al., 1994; Sinha-Hikim et al., 2003; Kamanga-Sollo et al., 2008), reduced differentiation (Doumit et al., 1996), and increased DNA accumulation (Snochowski et al., 1981; Arnold et al., 1997).

Androgen receptor mRNA expression

The biological action of androgen is mediated through the androgen receptor (AR), a member of the steroid hormone receptor family, which demonstrates its mediating function by binding to specific DNA sequences that influence transcription of androgen-responsive genes (Gelmann, 2002; Heinlein and Chang, 2002). *AR* gene expression data in the three muscles classified by sex and age are shown in Table 3. *AR* mRNA levels in the three muscles of barrows were all lower than those of boars at 147 and 210 d of age (p<0.05). In our study, *AR* mRNA levels in the skeletal muscles decreased after castration, which is consistent with a previous report in rats (Antonio et al., 1999). Several studies performed in humans (Kadi et al., 2000; Bamman et al., 2001; Sinha-Hikim et al., 2004)

and animals (Doumit et al., 1996; Jordan et al., 1997; Mateescu and Thonney, 2002) have also shown that the mRNA levels of the *AR* gene are up-regulated in muscle following androgen treatment. Also, *AR* gene mRNA levels are up-regulated in satellite cells following androgen administration (Doumit et al., 1996; Sinha-Hikim et al., 2004), which may potentially enhance the sensitivity of satellite cells to androgen treatment.

AR and its co-regulatory proteins are pivotal factors in skeletal muscle differentiation (Wannenes et al., 2008). Hickson (1985) showed that skeletal muscle hypertrophy results from an increase of *AR* expression. Also, *AR* expression may be linked to allometric muscle growth patterns in cattle and compensatory gain in steers (Brandstetter et al., 2000). Lee (2002) showed that the androgen-AR signaling pathway may suppress myoblast cell growth and accelerate myoblast cell differentiation via enhanced myogenin expression. Thus, the higher *AR* mRNA levels in the skeletal muscles of intact males could increase the number of androgen binding sites and promote skeletal muscle growth. The results of the present study indicate that down-regulation of *AR* mRNA levels by castration could be a pathway by which castration affects skeletal muscle development.

Characterization of porcine *IGF-I* splice variants

There are two splice variants of the *IGF-I* gene expressed in skeletal muscle, *IGF-I Ea* and *MGF*. *IGF-I Ea* is similar to the hepatic endocrine type of *IGF-I*, and *MGF* is reported to be expressed in normal muscle and is markedly up-regulated after mechanical stimulation. Both splice variants possess the same coding region as the mature IGF-I. *MGF* mRNA is derived from the *IGF-I* gene by alternative precursor mRNA splicing, the sequence of which

Table 3. *Androgen receptor* (*AR*) gene mRNA levels in boars and barrows at 147 and 210 d of age

Muscle	147 d			210 d		
	Boars (n = 7)	Barrows (n = 7)	p-value	Boars (n = 7)	Barrows (n = 7)	p-value
BR	4.07±0.34	2.64±0.30	0.012	5.39±0.72	2.91±0.26	0.008
L	2.85±0.12	2.30±0.22	0.037	3.63±0.41	2.76±0.22	0.043
ST	3.14±0.34	2.54±0.15	0.048	3.36±0.24	2.66±0.26	0.018

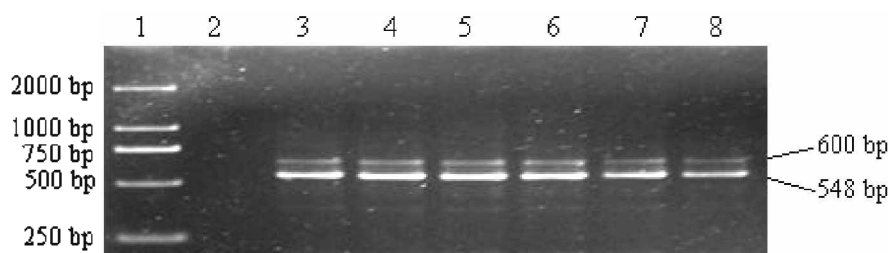BR = Brachialis, L = Longissimus, ST = Semitendinosus.

Figure 2. Amplification of the splice variants of *insulin-like growth factor-I* (*IGF-I*) gene. Lane 1, 2 kb DNA marker; lane 2, PCR amplification negative control; lanes 3, 4, 5, 6, 7 and 8, PCR amplification of the porcine splice variants of the *IGF-I* gene. PCR products of 548 bp and 600 bp corresponded to *IGF-I Ea* and *mechano growth factor* (*MGF*), respectively.

has a 49-base pair insertion in humans (Hameed et al., 2003) and a 52-base pair insertion in rodents (Hill and Goldspink, 2003) within the E domain.

In the current study, both *IGF-I* splice variants were detected in porcine skeletal muscle (Figure 2). Sequence analysis revealed that the PCR products of 548 bp and 600 bp corresponded to *IGF-I Ea* and *MGF*, respectively. The two types of cDNA sequences share the same coding region of the mature IGF-I, but differ by the presence (MGF) or absence (IGF-I Ea) of a 52 bp insert within the E domain (Figure 3). This results in a different carboxy terminal sequence.

IGF-I Ea and MGF mRNA expressions

Although insulin-like growth factor-I (IGF-I) production induced by growth hormone in liver is the major source of circulating IGF-I, local IGF-I production within skeletal muscle appears to play a more important role in skeletal muscle growth and maintenance (Sjögren et al., 1999; Yakar et al., 1999). It has been shown that androgens may exert an effect on skeletal muscle growth through local IGF-I in the human (Ferrando et al., 2002), rat (Lewis et al., 2002) and sheep (Mateescu and Thonney, 2005). However, two splice variants of the *IGF-I* gene are expressed in skeletal muscle. Different *IGF-I* splice variants may have

Figure 3. Alignment of the porcine splice variants of *insulin-like growth factor-I* (*IGF-I*) gene. The two types of cDNA sequences differ by the presence (*mechano growth factor, MGF*) or absence (*IGF-I Ea*) of a 52 bp insert (marked by ···).

Table 4. *IGF-I Ea* and *mechano growth factor (MGF)* mRNA levels in boars and barrows at 147 and 210 d of age

Gene	Muscle	147 d			210 d		
		Boars (n = 7)	Barrows (n = 7)	p-value	Boars (n = 7)	Barrows (n = 7)	p-value
IGF-I Ea	BR	8.68±0.42	7.69±0.57	0.103	8.60±0.43	6.23±0.88	0.003
	L	7.94±0.40	6.87±0.65	0.121	4.70±0.36	3.89±0.34	0.047
	ST	5.41±0.43	5.10±0.42	0.362	6.64±0.56	5.00±0.35	0.023
MGF	BR	3.91±0.64	2.91±0.18	0.100	4.43±0.66	2.44±0.37	0.010
	L	2.45±0.14	2.29±0.11	0.231	2.39±0.20	2.00±0.20	0.043
	ST	2.86±0.15	2.65±0.22	0.513	2.55±0.17	1.91±0.14	0.011

BR = Brachialis, L = Longissimus, ST = Semitendinosus.

different actions. The previous studies mentioned above did not differentiate between the expressions of *IGF-I Ea* and *MGF*.

Although overexpression of either *IGF-I Ea* (Barton-Davis et al., 1998) or *MGF* (Goldspink, 2005) increases muscle fibre size and strength, the two splice variants could be different growth factors and could be differentially regulated. *MGF* appears to be more acutely responsive to mechanical load, while *IGF-I Ea* up-regulation may occur more slowly and last longer (Petrella et al., 2006). Murine C2/C12 cells transfected with *MGF* expression plasmids showed increased proliferation of mononucleated myoblasts, but inhibited terminal differentiation into myotubes, whereas the *IGF-I Ea* peptide increased the mitotic index and also enhanced terminal differentiation (Yang and Goldspink, 2002). More recently, Mills (2007) demonstrated that MGF can promote myogenic cell migration.

The expression of *IGF-I Ea* and *MGF* classified by sex and age is shown in Table 4. We found that both *IGF-I* splice variants, which are potent mediators of skeletal muscle development, appeared to be similarly affected by castration. In the group slaughtered at 147 d, there were no significant differences in the mRNA levels of the two splice variants between boars and barrows (p>0.05). The expression of the two splice variant mRNAs in barrows, however, were significantly lower than in boars (p<0.05) in the 210 d group. Growth and carcass evaluations showed that carcass weight and lean meat yield were similar between boars and barrows at 147 d of age, whereas barrows had lower carcass weights and less lean meat yields at 210 d of age. Down-regulation of the expression of the two *IGF-I* gene splice variants may be an important

pathway by which castration affects skeletal muscle development.

AR, as a transcription factor, may regulate *IGF-I* gene expression. In our study, *AR* mRNA levels were affected by castration at both 147 and 210 d of age, whereas mRNA levels of two *IGF-I* splice variants were influenced only at 210 d of age. We speculate that down-regulation of the *AR* gene may occur earlier than the response of *IGF-I* (including *IGF-I Ea* and *MGF*) in gene transcription activity to castration. Further studies are required to confirm this speculation.

Myostatin mRNA expression

Myostatin, a member of the transforming growth factor beta (TGF-β) superfamily, is a specific negative regulator of skeletal muscle development and growth. Null mutations (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997; Gan et al., 2008) and gene knockout (McPherron et al., 1997) of the *myostatin* gene result in significant increases in muscle mass. Also, a T→A mutation in the promoter region of porcine *myostatin* gene has a positive effect on birth weight (Jiang et al., 2002).

The data for *myostatin* gene expression in the three muscles classified by sex and age are shown in Table 5. In a previous study, putative androgen response elements were identified in the 5'-regulatory region of the human *myostatin* promoter (Ma et al., 2001). It has also been reported that male transgenic mice over-expressing *myostatin* had reduced muscle mass, whereas female transgenic mice generated in the same way did not differ from wild-type (Reisz-Porszasz et al., 2003). Thus, the *myostatin* gene may be a direct target of the androgens. Moreover, the expression of *myostatin* gene might be

Table 5. *Myostatin* gene mRNA levels in boars and barrows at 147 and 210 d of age

Muscle	147 d			210 d		
	Boars (n = 7)	Barrows (n = 7)	p-value	Boars (n = 7)	Barrows (n = 7)	p-value
BR	1.72±0.24	2.02±0.35	0.112	2.12±0.28	2.35±0.18	0.390
L	2.04±0.25	1.70±0.29	0.469	2.33±0.16	2.76±0.29	0.260
ST	2.13±0.35	2.38±0.20	0.500	3.34±0.23	3.29±0.27	0.838

BR = Brachialis, L = Longissimus, ST = Semitendinosus.

regulated by some other hormone. Recently, Ma (2009) demonstrated that *myostatin* gene played a role in a metabolic process in muscle that was regulated by thyroid hormone levels. However, in this study, we found no significant differences in *myostatin* gene mRNA levels in the three muscles between boars and barrows at 147 and 210 d of age (p>0.05). Castration did not cause significant up-regulation of the *myostatin* gene mRNA levels in the skeletal muscles of male pigs, and our results are consistent with data obtained in sheep (Mateescu and Thonney, 2005). Nevertheless, *myostatin* might still play a role in the skeletal muscles of male pigs in response to castration. Because of the opposing actions of androgens and myostatin in skeletal muscle, it is tempting to speculate that androgen may suppress *myostatin* expression or action (Chen et al., 2005). Further studies are necessary to elucidate the speculation.

IMPLICATIONS

Our results indicate that castration of the male pig produces significant negative effects on skeletal muscle growth. Furthermore, we examined how the expression of *androgen receptor, IGF-1 Ea, mechano growth factor* and *myostatin* mRNA is regulated in the skeletal muscles of male pigs after castration. We suggest that locally expressed *androgen receptor, IGF-1 Ea* and *mechano growth factor* gene mRNA levels are down-regulated following castration, and that this may be one reason for the negative effects of castration on skeletal muscle development.

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